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Looking Under the Lamp Post: Neither *fruitless* nor *doublesex* Has Evolved to Generate Divergent Male Courtship in *Drosophila*

Jessica Cande,[1,2] David L. Stern,[2] Tomoko Morita,[2] Benjamin Prud'homme,[1,*] and Nicolas Gompel[1,3,*]
[1]Aix-Marseille Université, CNRS, Institut de Biologie du Développement de Marseille, UMR 7288, 13288 Marseille cedex 9, France
[2]Janelia Farm Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147, USA
[3]Ludwig Maximilians Universität München, Fakultät für Biologie, Biozentrum, Großhaderner Strasse 2, 82152 Planegg-Martinsried, Germany
*Correspondence: benjamin.prudhomme@univ-amu.fr (B.P.), gompel@biologie.uni-muenchen.de (N.G.)
http://dx.doi.org/10.1016/j.celrep.2014.06.023

SUMMARY

How do evolved genetic changes alter the nervous system to produce different patterns of behavior? We address this question using *Drosophila* male courtship behavior, which is innate, stereotyped, and evolves rapidly between species. *D. melanogaster* male courtship requires the male-specific isoforms of two transcription factors, *fruitless* and *doublesex*. These genes underlie genetic switches between female and male behaviors, making them excellent candidate genes for courtship behavior evolution. We tested their role in courtship evolution by transferring the entire locus for each gene from divergent species to *D. melanogaster*. We found that despite differences in Fru+ and Dsx+ cell numbers in wild-type species, cross-species transgenes rescued *D. melanogaster* courtship behavior and no species-specific behaviors were conferred. Therefore, *fru* and *dsx* are not a significant source of evolutionary variation in courtship behavior.

INTRODUCTION

Recent decades have seen enormous progress in our understanding of the molecular causes of evolutionary diversity, especially with regard to the evolution of morphology and physiology (Fletcher et al., 2001; Stern and Orgogozo, 2009). Often, genes that participate directly in the patterning or differentiation of tissues are the targets of evolutionary changes that alter the morphology of these tissues (Ronshaugen et al., 2002; Shapiro et al., 2004; Zhang et al., 2013). There also appears to be a strong bias during evolution toward genetic changes that alter gene-expression levels and patterns, rather than substitutions in protein-coding regions (Carroll, 2008; Levine and Tjian, 2003). This is likely because changes in expression patterns can alter specific aspects of morphology without deleterious pleiotropic effects (Stern, 2000). Our current understanding has been gained largely through studies of candidate genes (Davis and Patel, 2002; Lynch and Roth, 2011), and studies employing unbiased mapping of the genetic causes of morphological diversity have come to essentially the same conclusions (e.g., Shapiro et al., 2004; Sucena and Stern, 2000). Thus, candidate-gene approaches can provide valuable insights into the mechanisms of phenotypic evolution.

It is tempting to transfer the candidate-gene approach to studies of behavioral evolution. Behavior is generated by the activity of neural circuits, which are anatomical structures patterned by developmental genes and produced by developmental processes. Thus, genes that regulate the development and activity of these circuits would be excellent candidates for behavior evolution. Male courtship behavior in *Drosophila* species is an attractive target for such studies because not only do *Drosophila* species display extensive variation in courtship behavior (Markow and O'Grady, 2005; Spieth, 1952), but the two genes that act as master regulators of sexual behavior have also been identified in *D. melanogaster.*

Courtship in *Drosophila* consists of multiple relatively stereotypical behavioral modules and includes signaling between males and females in multiple modalities, including visual, auditory, somatosensory, and gustatory inputs (Hall, 1994; Krstic et al., 2009). In *D. melanogaster*, apparently all male sex-specific behaviors are regulated by the activity of two transcription factors, *doublesex* (*dsx*) and *fruitless* (*fru*), which are alternatively spliced in males and females (Baker and Wolfner, 1988; Burtis and Baker, 1989; Demir and Dickson, 2005; Kimura et al., 2008; Manoli et al., 2005; Rideout et al., 2007). *dsx* is required for all external sexually dimorphic features (Hildreth, 1965), as well as some male-specific behaviors such as courtship initiation and song (Kimura et al., 2008; Rideout et al., 2007; Villella and Hall, 1996). Male-specific Fruitless (Fru^M) is expressed in ~1,200 neurons in the CNS and in some peripheral sensory neurons (Lee et al., 2000; Manoli et al., 2005), all of which appear to be involved in courtship behavior or sensing courtship-related cues (e.g., Datta et al., 2008; Kimura et al., 2008; Stockinger et al., 2005). Elimination of Fru^M function leads to the complete abrogation of male courtship (Ito et al., 1996; Ryner et al., 1996), whereas ectopic expression of Fru^M in females is sufficient to induce male courtship behavior (Demir and Dickson, 2005; Manoli et al., 2005). The functional dissection of

populations of Fru^M- and Dsx^M-positive neurons led to the identification of neurons in the brain that are required to activate male sexual behavior (Kimura et al., 2008) and neurons in the thorax that drive specific subsets of sexual behavior (Clyne and Miesenböck, 2008; Rideout et al., 2007). At a finer level, an individual Fru^M-positive neuron and a single Dsx^M-positive muscle together are required to produce a single element of courtship song (Shirangi et al., 2013), suggesting that the neuroanatomical substrate regulated by *fru* and *dsx* is highly modular. Studies of morphological evolution have suggested that modularity can contribute to more rapid evolution of new phenotypes (Stern and Orgogozo, 2009; Wagner et al., 2007). Here, we test the hypothesis that *D. melanogaster* courtship has evolved through changes in the *fru* and *dsx* loci.

RESULTS AND DISCUSSION

Candidate Loci for Cross-Species Transfer of Courtship

We surveyed drosophilid species with genome resources (Clark et al., 2007) and then chose three species whose courtship is qualitatively different from that of *D. melanogaster*. In *D. melanogaster*, males first tap the female and give chase, alternating chasing with bouts of unilateral wing extension (singing) and other behaviors such as abdomen curling and licking (Hall, 1994). While courtship in both *D. yakuba* and *D. persimilis* superficially resembles that of *D. melanogaster*, these species also incorporate unique, species-specific elements (Spieth, 1952). *D. yakuba* males often circle the female slowly while shaking both wings (Figure 1A; Movie S1). Likewise, *D. persimilis* males punctuate unilateral wing extensions by running in front of the female and extending their proboscis while pumping their abdomen up and down, and usually will also simultaneously hold out their wings, stomp, or wave their T1 legs (Figure 1A; Movie S1). *D. ananassae* males alternate frequent, short (~1/2 s), bilateral, low-angle wing vibrations with lunges at the female and abdomen curling (Singh and Singh, 2003; Figure 1A; Movie S1). Further, all four species differ significantly in their courtship song (Demetriades et al., 1999; Markow and O'Grady, 2005; Waldron, 1964; Yamada et al., 2002).

The expression patterns of Fru^M and Dsx differ subtly among these fly species in the number of cells present in specific clusters in the brain ((Usui-Aoki et al., 2005; Figures S1A–S1F). In addition, both loci contain large introns and upstream noncoding regions (Figures 1B and 1C), which suggests that they contain extensive *cis*-regulatory regions (Nelson et al., 2004). Thus, any test of the role of *fru* and *dsx* in courtship evolution must encompass both protein coding and noncoding potential *cis*-regulatory sequences. We therefore cloned the entire *fru* locus from *D. ananassae* and *D. persimilis* (Figure 1B) and the entire *dsx* locus from *D. melanogaster*, *D. yakuba*, *D. ananassae*, and *D. persimilis* (Figure 1C), and made *D. melanogaster* transgenics using BAC recombineering and the P[acman] transgenesis system (Venken et al., 2006). We crossed these transgenes into a *D. melanogaster fru* or *dsx* null background, respectively. This effectively replaced the native *D. melanogaster* locus with a heterologous one from another species or with a *D. melanogaster dsx* transgene as a positive control. We were unable to get transformants for a *D. melanogaster fru*-positive control, likely due to the poor integration of constructs of this size (~200 kb; Figure 1B; Venken et al., 2009). All *dsx* transgenes rescued the $dsx^{\Delta}/dsx^{\Delta}$ intersex phenotype (Hildreth, 1965) with only minor defects in sex comb formation (Figure S2A). Furthermore, despite significant species-specific differences in sex combs and genital morphology (Figure S1G), all *dsx* rescue flies resembled the *D. melanogaster* wild-type (Figures S1G and S2A). Except for the *D. ananassae dsx* transgene, this rescue was sufficient to restore fertility (Figure S2B). This indicates that *dsx* by itself is not sufficient to specify species differences in sex combs or male genital morphology. The *D. ananassae* and *D. persimilis fruitless* transgenes likewise rescued the noncourting phenotype of hypomorphic *fru* mutant males and the pupal lethality of *fru* null mutants (Table S1; Figures 2B and 2C).

Visual Analysis of Courtship Behavior

When we analyzed courtship, we found that all of the transgenes were largely able to rescue the defects in courtship index (CI) seen in *D. melanogaster dsx* or *fru* mutants (Table S1). However, when we looked for qualitative changes in *D. melanogaster* male courtship in the transgenics, we found that none of the qualitative species differences depicted in Figure 1A were transferred. *D. melanogaster* males carrying heterologous transgenes in combination with *D. melanogaster dsx* or *fru* null mutations behaved qualitatively like any other *D. melanogaster* male (examples are shown in Movie S2).

Although the transgenes failed to transfer the most obvious species-specific elements of courtship behavior, we wondered whether they transferred subtle quantitative differences in courtship steps that are shared across the four species. Therefore, we quantified not only species-specific aspects of courtship, such as the slow circling seen in *D. yakuba*, but also shared aspects, such as the time spent with wings extended or the angle of the wing relative to the body axis (summarized in Table S1). Many of these behaviors are correlated with each other and we therefore performed a principal component analysis to identify the statistically independent elements of these behaviors (Joliffe, 1986).

The first two principal components together explain approximately 60%–80% of the variance in each of the three different species comparisons (Figure 2). Males of all three species (*D. yakuba*, *D. ananassae*, and *D. persimilis*) tend to separate from *D. melanogaster* males most strongly along the first principal-component axis (Figure 2). This separation is less strong for *D. persimilis*, probably because not all *D. persimilis* males performed the proboscis-extension/abdomen-pumping combination. Wild-type *D. persimilis* males that did not do this behavior clustered close to *D. melanogaster*, reflecting the overall similarity of their courtship. In all cases, the *dsx* and *fru* transgenics, either alone or in combination as transheterozygotes, as well as the $dsx^{\Delta}/dsx^{\Delta}$-negative control clustered with the *D. melanogaster* wild-type and not with the transgene species of origin. This indicates that the transgenes rescued wild-type *D. melanogaster* behavior and did not appear to transfer any quantitative aspects of male courtship behavior between species.

Analysis of Courtship Song

Courtship song provides a potentially more sensitive assay of species differences because multiple details of courtship song

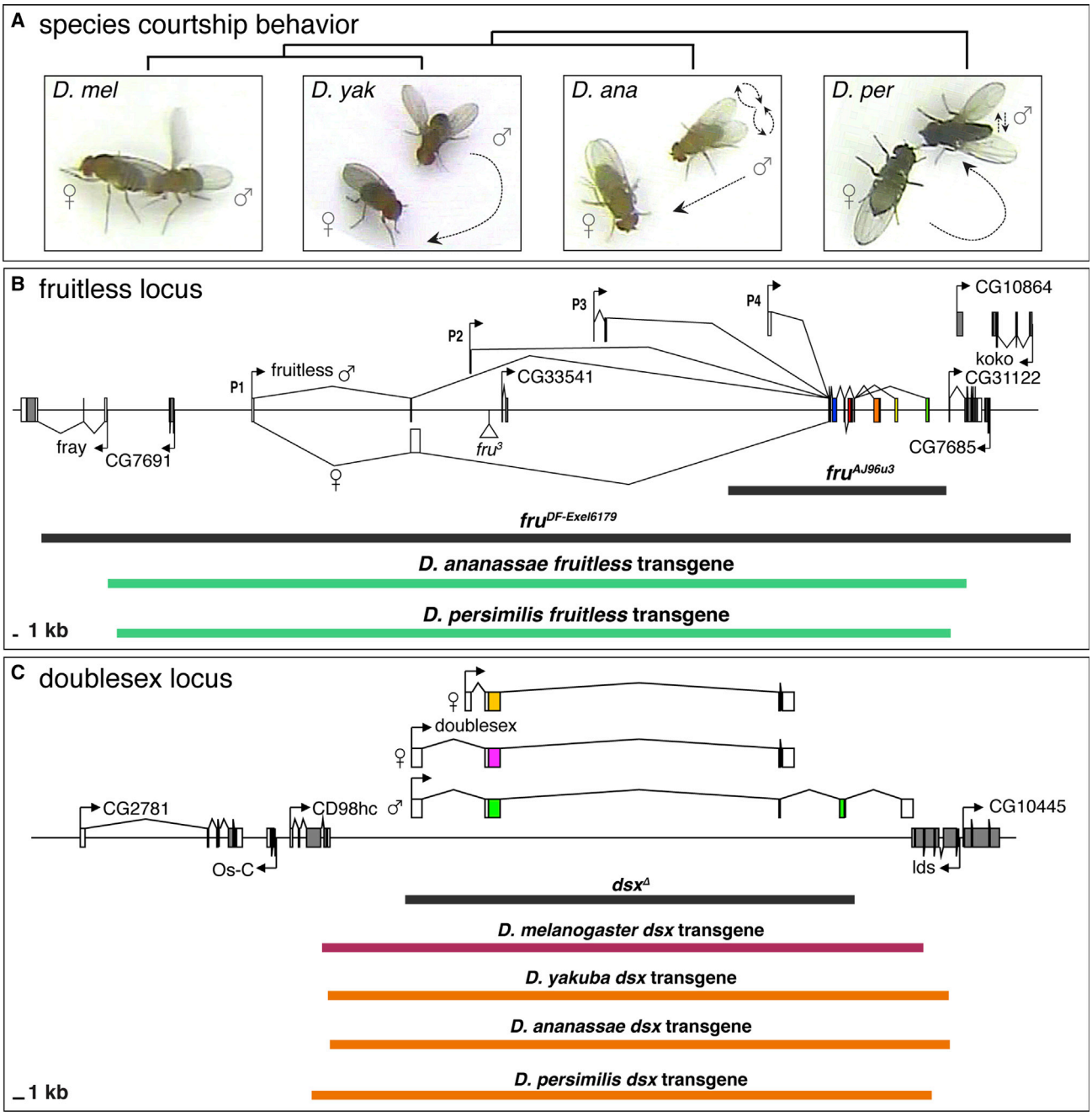

Figure 1. Candidate Species Courtship Behaviors and Candidate Genes

(A) From left to right: a *D. melanogaster* male licking and abdomen curling with wing extended, a *D. yakuba* male wing shaking and circling (arrow), a *D. ananassae* male singing (arrows indicate wing movements and lunges), and a *D. persimilis* running in front of the female (arrow) and extending his proboscis, holding his wings out, stomping, and pumping his abdomen up and down (small arrows).

(B) The *D. melanogaster fru* locus, showing the *D. ananassae* and *D. persimilis fru* transgenes (green bars), the two deletions ($fru^{DF\text{-}Exel6179}$ (Parks et al., 2004) and fru^{AJ96u3} (Song et al., 2002), gray bars), and the location of the fru^{3} *P*-element insertion (Goodwin et al., 2000).

(C) The *D. melanogaster dsx* locus, showing the *dsx* transgenes. Red bar, *D. melanogaster*; orange bars, cross-species transgenes; gray bar, *dsx* deletion (FDD-0250107; Parks et al., 2004).

See also Figure S1 and Movie S1.

are quantifiable and variable between species (Markow and O'Grady, 2005). The four species we studied sing songs that differ qualitatively and quantitatively, which is easily seen in the song traces (Figure 3A) and heard in the audio files (Movie S3). *D. melanogaster* sings with alternating bouts of sine and pulse song (von Schilcher, 1976). *D. yakuba* sings with pulse and clack

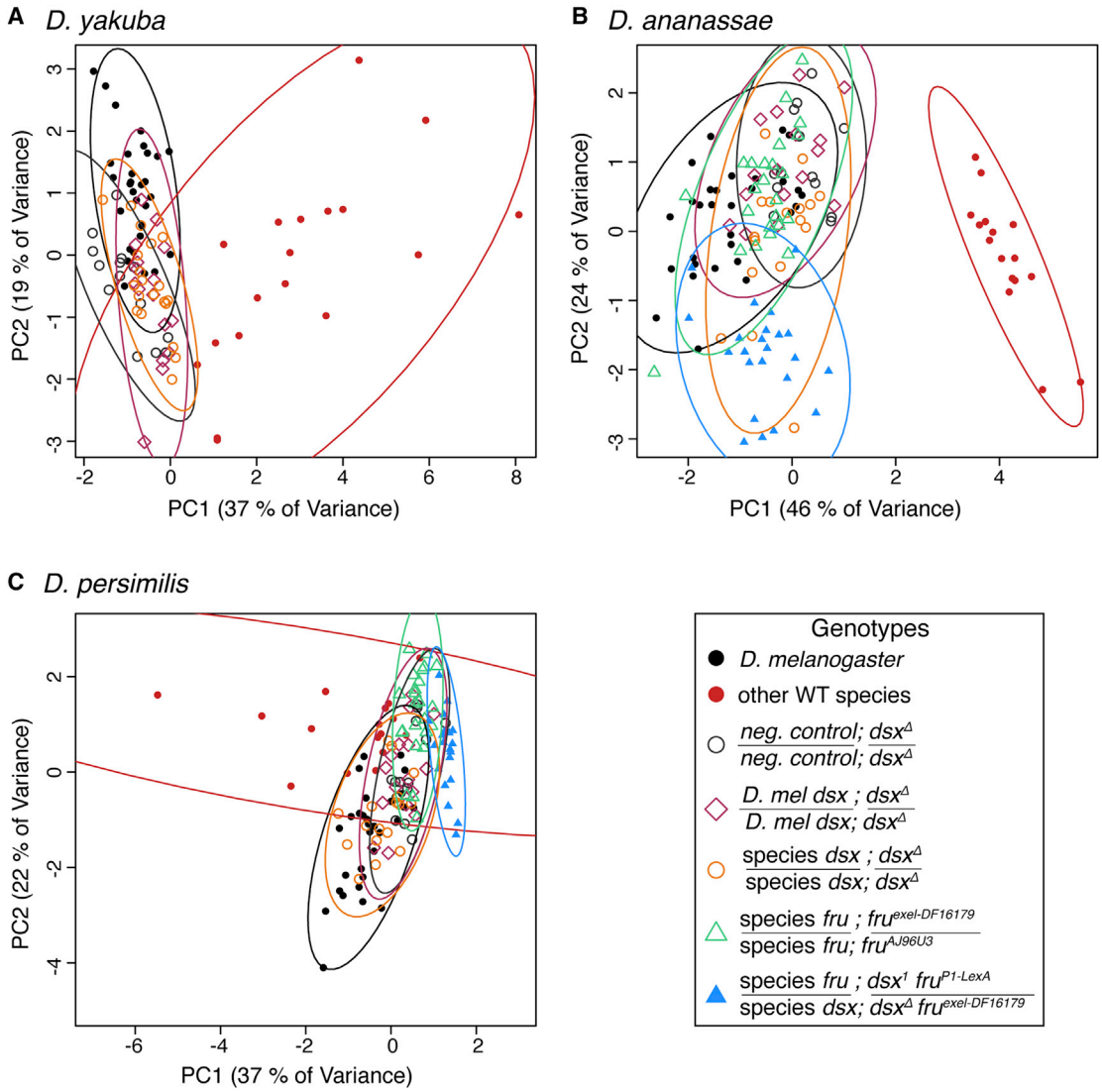

Figure 2. Principal Component Analysis of Courtship in Wild-Type Species and Heterologous *dsx* and *fru* Transgenics

(A–C) The data are divided by species. In each panel, males from the wild-type transgene species of origin (*D. yakuba* [A], *D. ananassae* [B], and *D. persimilis* [C]) are plotted in filled red circles and the *D. melanogaster* wild-type is plotted in filled black circles. Species-specific transgenes in an endogenous *D. melanogaster* mutant background are plotted as described by the genotype key at the lower right. $fru^{AJ96u3}/fru^{DF\text{-}exel6179}$ males carry a transheterozygous deletion and are *fru* null; 95% confidence ellipses are indicated.

See also Figures S1 and S2, Table S1, and Movie S2.

song with a much longer interevent interval than *D. melanogaster* (Demetriades et al., 1999). *D. persimilis* sings polycyclic pulses with an interpulse interval approximately twice as long as that in *D. melanogaster* (Noor and Aquadro, 1998). *D. ananassae* sings short bursts of polycyclic buzzes (Yamada et al., 2002). All transgenic males, with the exception of the *D. persimilis dsx/fru* transheterozygote in a double-mutant background, sing like *D. melanogaster*, with alternating bouts of pulse and sine song (Figure 3B; Movie S3). The *D. persimilis dsx/fru* transheterozygous transgenic flies sing normal-pulse song, but not sine song. In *D. melanogaster*, *dsx* and *fru* act in concert to regulate sine song (Shirangi et al., 2013). It is possible that the *D. persimilis dsx/fru* trangenes are weak hypomorphic alleles that in combination fail to complement redundant *dsx* and *fru* sine song functions. Regardless, there was no evidence for species-specific song interconversion. In all cases, even the quantitative features of song, such as the interpulse interval (Figure 3), resembled those of *D. melanogaster* song.

CONCLUSIONS

We tested the hypothesis that elements of species-specific courtship behavior are encoded by the two known master-control genes for courtship behavior in *D. melanogaster*, *doublesex*

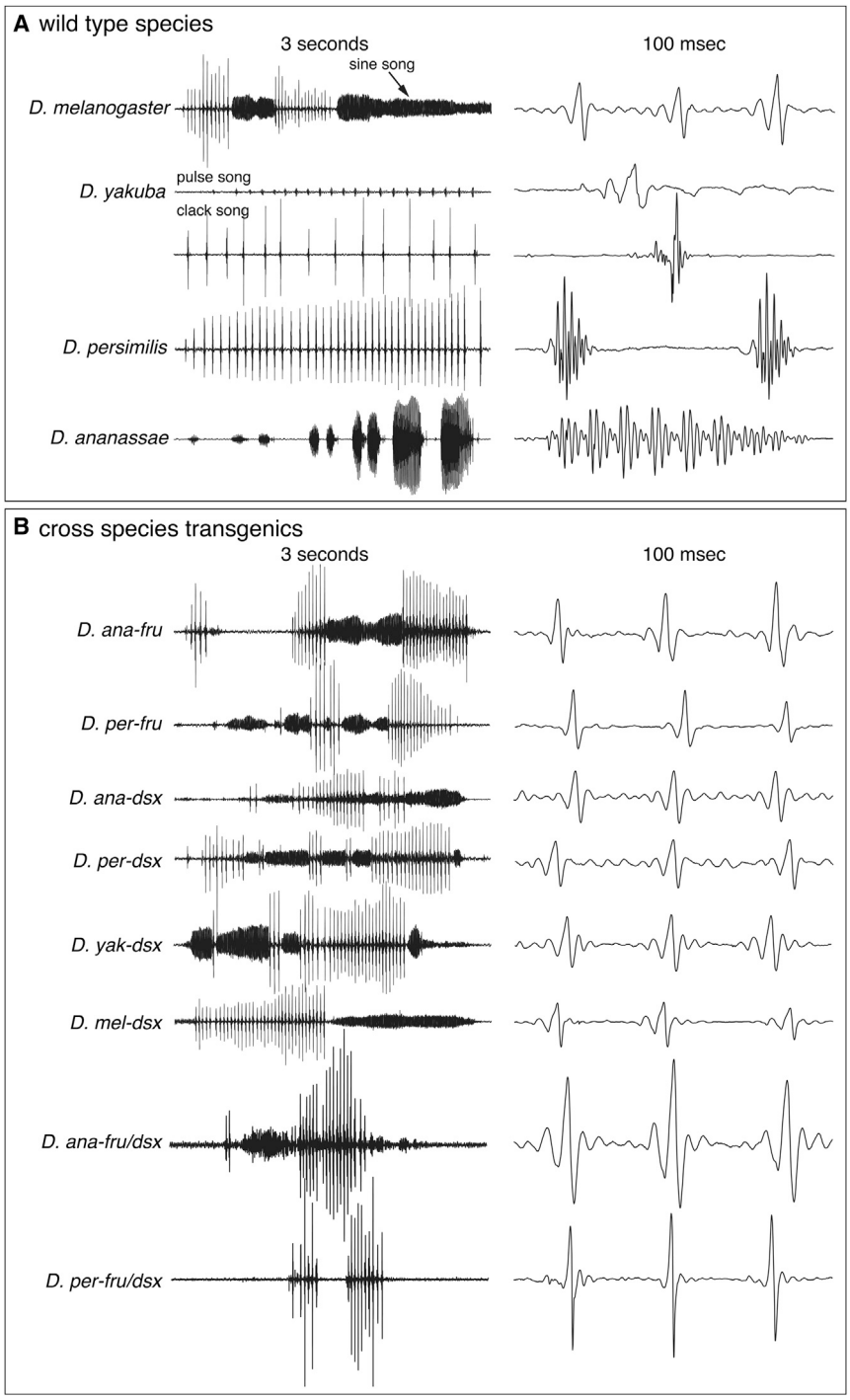

Figure 3. Courtship Song in Wild-Type Species and *dsx* and *fru* Transgenics

(A) The four wild-type species.

(B) The *dsx* and *fru* transgenics, with the same genotypes as in Figure 2.

In both panels, a 3 s interval is shown at left and a 100 ms interval of representative song pulses (or pulses and clacks, *D. yakuba* [A]) is shown at right. See also Figures S1 and S2 and Movie S3.

and *fruitless*. We tested the role of both coding and *cis*-regulatory regions by performing BAC transgenesis of the entire *dsx* and *fru* genes from each of three species into *D. melanogaster* flies deficient for each of the native loci. Our experiments revealed that all of these transgenes were sufficient to rescue courtship behavior to near wild-type levels and that all transgenes rescued normal *D. melanogaster* courtship behavior. We found no evidence that any qualitative or quantitative details of courtship behavior were transferred between species.

This result stands in stark contrast to a large and growing body of evidence that suggests that the evolution of candidate genes often contributes to morphological diversity (e.g., Prud'homme et al., 2006; Tomoyasu et al., 2009). Unfortunately, no other strong candidate genes for courtship behavior are currently available that would allow us to test this hypothesis further. Instead, other approaches are required to unravel the genetic changes that underlie behavioral diversity. While the *fru* and *dsx* genes themselves are not substrates for courtship evolution in *D. melanogaster* species, the same cannot be said about Fru^M- and Dsx-positive neurons. It may be possible to leverage the genetic tools developed for these genes in *D. melanogaster* to examine the function of these cells in other *Drosophila* species using next-generation genome editing tools such as CRISPR (Gratz et al., 2013). Another approach is the genetic mapping of strain and species differences (Cande et al., 2012; Gould et al., 2010; Kitano et al., 2009). However, current methodologies provide only limited resolution of the genomic regions that contribute to behavior differences (Mackay, 2009), and identification of the causal genes remains a significant challenge.

EXPERIMENTAL PROCEDURES

Fly Stocks and Crosses

The wild-type species stocks used for quantitative analysis corresponded to the sequenced genomes and BAC resources and are available from the San Diego species stock center under the following stock numbers: *D. yakuba* #14021-0261.01, *D. ananassae* #14024-0371.13, and *D. persimilis* #14011-0111.49 and #14011-0111.50. Species-specific behaviors were confirmed with multiple additional lines (*D. ananassae* #14024-0371.16, #14024-0371.22, and #14024-0371.34; *D. persimilis* #14011-0111.41; and additional *D. yakuba* lines described in Cande et al. [2012]). The *D. melanogaster* wild-type used was Canton S. Generation of the custom *dsx* deletion used in the transgene rescue male flies is described below. fru^3 is a *P*-element insertion near the P1

promoter described in Goodwin et al. (2000) and the $fru^{DF\text{-}Exel6179}$ deletion is described in Parks et al. (2004). The fru^{AJ96u3} allele (Song et al., 2002) and dsx^{1} fru^{P1lexA} (Hildreth, 1965; Mellert et al., 2010) recombinants were a generous gift from Bruce Baker's lab. All transgenes were inserted into the VK16 AttP site on chromosome 2 (Venken et al., 2006), and all *fru* and/or *dsx* alleles were balanced over TM6B. A modified Stinger (Barolo et al., 2000) construct with an AttB site and mini-white marker was knocked into the same VK16 site and used as a negative control. All crosses and species were raised on cornmeal-agar media at 20°C–22°C according to standard methods.

Generation of the *fruitless* and *doublesex* Transgenics

Filters spotted with the BAC libraries for *D. yakuba*, *D. ananassae*, and *D. persimilis* (DY Ba, DA Ba, and DP Ba, respectively, from Arizona Genomics Institute) were probed via standard Southern hybridization with ~500 bp probes labeled through random primer extension by incubating 50 ng template DNA in the presence of Klenow and random hexamers to incorporate P^{32} deoxycytidine 5′-triphosphate. Labeled filters were analyzed via phosphoimaging on a Fujifilm FLA-5000 phosphoimager. *D. yakuba dsx* (coordinates 3R: 6474025-6528771, Flybase release R1.3, cloned from the BAC clone at address 27I2), *D. ananassae dsx* (scaffold_13340: 14858455-14908776, clone address 21E22), *D. ananassae fru* (scaffold_13340: 9713578-9867775, clone address 20H8), *D. persimilis dsx* (scaffold 0: 3349345-3409770, clone address 31M5), and *D. persimilis fru* (scaffold 0: 8550968-8730455, clone address 36N18) were cloned from the BAC library clones into the P[acman] transgenesis vector using BAC recombineering (Venken et al., 2006). *D. melanogaster dsx* (3R: 3749186-3800800) was recombineered from RPCI-98 (CHORI) clone 36E18. Recombineered clones were checked for proper left- and right-hand integration into P[acman] via PCR, and were also digested with clone-appropriate restriction enzymes and run on a pulse field gel prior to injection. VK16 flies were injected with 100 ng/μl transgene DNA that had been midi-prepped from 100 ml of culture induced for 4–6 hr with 0.1% arabinose and purified using a Nucleobond Xtra midi kit (reference 740410.50; Machery-Nagel). DNA preps were stored at 4°C and injected in the next 3 days.

Generation of the *D. melanogaster dsx* Deletion

The FDD-0250107 deletion (Parks et al., 2004) was generated by placing the f00683 and d06446 FRT insertions in *trans* in combination with an HS-FLP on X (BSC1929) and heat shocking as described previously (Parks et al., 2004). Backcross progeny were screened for the presence of the deletion using two-sided PCR with the following primers:

WHXPleft 5′ - cctcgatatacagaccgataaaac-3′
WHXPRight 5′-tactattcctttcactcgcacttattg-3′
dsxDelF 5′-TGTAGTTGGCCAGGATTAGTGAGC-3′
dsxDelR 5′-TGAGTGGTTCGACCTATATCGTC-3′

dsx^{Δ} homozygous flies had an intersex phenotype consistent with previously described *dsx* null mutations (Hildreth, 1965). A dsx^{Δ} fru^{Δ} double-mutant chromosome was generated by recombining the new *dsx* deletion with $fru^{DF\text{-}Exel6179}$ and screening for recombinants using the *dsx* primers dsxDelF and WHXPleft, and the $fru^{DF\text{-}Exel6179}$-specific primers XP5′minus 5′-TTTACTC CAGTCACAGCTTTG-3′ (Parks et al., 2004) and frudel3′R 5′-ACACGATCAT GTGCAACTGATAAG-3′.

Visual Analysis of Courtship Behavior

Flies were kept at 20°C–22°C, 50% humidity, and constant 12 hr day/night cycle. Male flies were isolated upon eclosion using a light application of CO_2, aged to 4–6 days (except for *D. persimilis*, which were aged 5–7 days), and aspirated into plexiglass courtship chambers with a hemispherical well 15 mm in diameter and 3 mm deep. Virgin females were handled similarly except that they were kept in groups of up to 50 flies. All movies were shot on a JVC color video camera TK-C1481BEG mounted on a Leica Z6 APO zoom system. Video capture was performed using Pinnacle Video Capture 1.0.1 software (Elgato Systems) set at 640 × 480 pixels, 25 fps. All movies were shot in the first 3 hr after dawn and lasted 30 min or until copulation occurred, except for the wild-type *D. yakuba* movies, which were taken from (Cande et al., 2012) and are 15 min long.

All *D. melanogaster* wild-type and BAC transgene flies were pooled into three lots, randomized, and scored blind. This could not be done with the other wild-type species because they are readily distinguishable based on external morphology. Video analysis was done using Annotation 1.0. The CI (time spent courting / total time filmed) was calculated for the full video and the other parameters were calculated from the first 10 min. We calculated the wing extension index (WEI) as the time spent courting with wing(s) extended / time spent courting, wing extension frequency as the total number of wing extension events / time spent courting (likewise with abdomen curling frequency), and wing extension duration as the average length of time (in seconds) of all wing extension events per fly. to To exclude flicks and scissoring from the analysis, extremely short wing extensions (<½ s) were not counted, except for the *D. ananassae* wild-type species, in which nearly all wing extensions were <½ s. Wing angle was measured by drawing one line through the center of the fly and another from the tip of the extended or contralateral wing through the wing hinge, and connecting it to the line through the fly's center. The angle between these two lines was measured in Illustrator, and for each fly ten different wing extensions were measured at their maxima and averaged. The *D. yakuba* species-specific behavior of wing shaking while slowly circling was broken into its two components and each was scored separately. The *D. persimilis* behavior of running in front of the female, extending the proboscis, abdomen pumping, holding the wings out, and stomping was scored both in its entirety and for the proboscis extension alone (without licking), as this was an easily recognizable signature of this behavior. All species-specific behaviors were normalized to the total time spent courting and 16–30 males were scored for each species or genotype, with the exceptions of the $fru^{3}/fru^{DF\text{-}exel6179}$-negative control (12 males) and the *D. persimilis fru*/ *D. per fru*; $fru^{3}/fru^{DF\text{-}Exel6179}$ transgenic (14 males). Males that had a CI < 0.1 or spent <100 s courting were dropped from additional analysis after the CI was calculated. This included ~50% of the $dsx^{\Delta}/dsx^{\Delta}$-negative control individualsthat presumably were XX rather than XY intersexes. All behavior data analysis and plotting were done in R (R Development Core Team, 2013). Principal component analysis was carried out using the prcomp function with centering and scaling, and 95% confidence ellipses were calculated using dataEllipse. Species-specific variables, i.e., slow circling and wing shaking (*D. yakuba*), and proboscis extensions (excluding licks) and the proboscis-extension/abdomen-pumping/stomping combination (*D. persimilis*), that were not performed by the species under comparison were not included in a given principal component analysis.

Analysis of Courtship Song

Courtship song was recorded and analyzed as described previously (Arthur et al., 2013).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, one table, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.023.

AUTHOR CONTRIBUTIONS

J.C., B.P., and N.G. generated the transgenic flies. J.C. recorded and analyzed courtship videos. T.M. and D.S. recorded and analyzed song.

ACKNOWLEDGMENTS

We thank Serge Alonso for his help in probing the BAC filters, Justine Magrina for embryo injections, and Michelle Arbeitman and Ilona Grunwald for anti-Dsx and anti-Dlg antibodies, respectively. We acknowledge Flybase for information support. This work was supported by an HFSP long-term fellowship (LT000708/2010-L to J.C.), FSER, FRM, EURYI, CNRS, France-BioImaging/PICsL (ANR-10-INSB-04-01, "Investissements d'Avenir"), and the Howard Hughes Medical Institute.

Received: February 28, 2014
Revised: May 13, 2014
Accepted: June 17, 2014
Published: July 10, 2014

REFERENCES

Arthur, B.J., Sunayama-Morita, T., Coen, P., Murthy, M., and Stern, D.L. (2013). Multi-channel acoustic recording and automated analysis of Drosophila courtship songs. BMC Biol. *11*, 11.

Baker, B.S., and Wolfner, M.F. (1988). A molecular analysis of doublesex, a bifunctional gene that controls both male and female sexual differentiation in Drosophila melanogaster. Genes Dev. *2*, 477–489.

Barolo, S., Carver, L.A., and Posakony, J.W. (2000). GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. Biotechniques *29*, 726, 728, 730, 732.

Burtis, K.C., and Baker, B.S. (1989). Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell *56*, 997–1010.

Cande, J., Andolfatto, P., Prud'homme, B., Stern, D.L., and Gompel, N. (2012). Evolution of multiple additive loci caused divergence between Drosophila yakuba and D. santomea in wing rowing during male courtship. PLoS ONE *7*, e43888.

Carroll, S.B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. Cell *134*, 25–36.

Clark, A.G., Eisen, M.B., Smith, D.R., Bergman, C.M., Oliver, B., Markow, T.A., Kaufman, T.C., Kellis, M., Gelbart, W., Iyer, V.N., et al.; Drosophila 12 Genomes Consortium. (2007). Evolution of genes and genomes on the Drosophila phylogeny. Nature *450*, 203–218.

Clyne, J.D., and Miesenböck, G. (2008). Sex-specific control and tuning of the pattern generator for courtship song in Drosophila. Cell *133*, 354–363.

Datta, S.R., Vasconcelos, M.L., Ruta, V., Luo, S., Wong, A., Demir, E., Flores, J., Balonze, K., Dickson, B.J., and Axel, R. (2008). The Drosophila pheromone cVA activates a sexually dimorphic neural circuit. Nature *452*, 473–477.

Davis, G.K., and Patel, N.H. (2002). Short, long, and beyond: molecular and embryological approaches to insect segmentation. Annu. Rev. Entomol. *47*, 669–699.

Demetriades, M.C., Thackeray, J.R., and Kyriacou, C.P. (1999). Courtship song rhythms in Drosophila yakuba. Anim. Behav. *57*, 379–386.

Demir, E., and Dickson, B.J. (2005). fruitless splicing specifies male courtship behavior in Drosophila. Cell *121*, 785–794.

Fletcher, G.L., Hew, C.L., and Davies, P.L. (2001). Antifreeze proteins of teleost fishes. Annu. Rev. Physiol. *63*, 359–390.

Goodwin, S.F., Taylor, B.J., Villella, A., Foss, M., Ryner, L.C., Baker, B.S., and Hall, J.C. (2000). Aberrant splicing and altered spatial expression patterns in fruitless mutants of Drosophila melanogaster. Genetics *154*, 725–745.

Gould, F., Estock, M., Hillier, N.K., Powell, B., Groot, A.T., Ward, C.M., Emerson, J.L., Schal, C., and Vickers, N.J. (2010). Sexual isolation of male moths explained by a single pheromone response QTL containing four receptor genes. Proc. Natl. Acad. Sci. USA *107*, 8660–8665.

Gratz, S.J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K., Harrison, M.M., Wildonger, J., and O'Connor-Giles, K.M. (2013). Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics *194*, 1029–1035.

Hall, J.C. (1994). The mating of a fly. Science *264*, 1702–1714.

Hildreth, P.E. (1965). Doublesex, recessive gene that transforms both males and females of Drosophila into intersexes. Genetics *51*, 659–678.

Ito, H., Fujitani, K., Usui, K., Shimizu-Nishikawa, K., Tanaka, S., and Yamamoto, D. (1996). Sexual orientation in Drosophila is altered by the satori mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain. Proc. Natl. Acad. Sci. USA *93*, 9687–9692.

Joliffe, I.T. (1986). Principal Component Analysis (New York: Springer).

Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T., and Yamamoto, D. (2008). Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship. Neuron *59*, 759–769.

Kitano, J., Ross, J.A., Mori, S., Kume, M., Jones, F.C., Chan, Y.F., Absher, D.M., Grimwood, J., Schmutz, J., Myers, R.M., et al. (2009). A role for a neo-sex chromosome in stickleback speciation. Nature *461*, 1079–1083.

Krstic, D., Boll, W., and Noll, M. (2009). Sensory integration regulating male courtship behavior in Drosophila. PLoS ONE *4*, e4457.

Lee, G., Foss, M., Goodwin, S.F., Carlo, T., Taylor, B.J., and Hall, J.C. (2000). Spatial, temporal, and sexually dimorphic expression patterns of the fruitless gene in the Drosophila central nervous system. J. Neurobiol. *43*, 404–426.

Levine, M., and Tjian, R. (2003). Transcription regulation and animal diversity. Nature *424*, 147–151.

Lynch, J.A., and Roth, S. (2011). The evolution of dorsal-ventral patterning mechanisms in insects. Genes Dev. *25*, 107–118.

Mackay, T.F. (2009). The genetic architecture of complex behaviors: lessons from Drosophila. Genetica *136*, 295–302.

Manoli, D.S., Foss, M., Villella, A., Taylor, B.J., Hall, J.C., and Baker, B.S. (2005). Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. Nature *436*, 395–400.

Markow, T.A., and O'Grady, P.M. (2005). Evolutionary genetics of reproductive behavior in Drosophila: connecting the dots. Annu. Rev. Genet. *39*, 263–291.

Mellert, D.J., Knapp, J.M., Manoli, D.S., Meissner, G.W., and Baker, B.S. (2010). Midline crossing by gustatory receptor neuron axons is regulated by fruitless, doublesex and the Roundabout receptors. Development *137*, 323–332.

Nelson, C.E., Hersh, B.M., and Carroll, S.B. (2004). The regulatory content of intergenic DNA shapes genome architecture. Genome Biol. *5*, R25.

Noor, M.A.F., and Aquadro, C.F. (1998). Courtship songs of Drosophila pseudoobscura and D. persimilis: analysis of variation. Anim. Behav. *56*, 115–125.

Parks, A.L., Cook, K.R., Belvin, M., Dompe, N.A., Fawcett, R., Huppert, K., Tan, L.R., Winter, C.G., Bogart, K.P., Deal, J.E., et al. (2004). Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. Nat. Genet. *36*, 288–292.

Prud'homme, B., Gompel, N., Rokas, A., Kassner, V.A., Williams, T.M., Yeh, S.D., True, J.R., and Carroll, S.B. (2006). Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. Nature *440*, 1050–1053.

R Development Core Team (2013). R: a language and environment for statistical computing (Vienna, Austria: R Foundation for Statistical Computing).

Rideout, E.J., Billeter, J.C., and Goodwin, S.F. (2007). The sex-determination genes fruitless and doublesex specify a neural substrate required for courtship song. Curr. Biol. *17*, 1473–1478.

Ronshaugen, M., McGinnis, N., and McGinnis, W. (2002). Hox protein mutation and macroevolution of the insect body plan. Nature *415*, 914–917.

Ryner, L.C., Goodwin, S.F., Castrillon, D.H., Anand, A., Villella, A., Baker, B.S., Hall, J.C., Taylor, B.J., and Wasserman, S.A. (1996). Control of male sexual behavior and sexual orientation in Drosophila by the fruitless gene. Cell *87*, 1079–1089.

Shapiro, M.D., Marks, M.E., Peichel, C.L., Blackman, B.K., Nereng, K.S., Jónsson, B., Schluter, D., and Kingsley, D.M. (2004). Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature *428*, 717–723.

Shirangi, T.R., Stern, D.L., and Truman, J.W. (2013). Motor control of Drosophila courtship song. Cell Reports *5*, 678–686.

Singh, S.R., and Singh, B.N. (2003). Behavioral genetics of Drosophila ananassae. Genet. Mol. Res. *2*, 394–409.

Song, H.J., Billeter, J.C., Reynaud, E., Carlo, T., Spana, E.P., Perrimon, N., Goodwin, S.F., Baker, B.S., and Taylor, B.J. (2002). The fruitless gene is required for the proper formation of axonal tracts in the embryonic central nervous system of Drosophila. Genetics *162*, 1703–1724.

Spieth, H.T. (1952). Mating behavior within the genus Drosophila (Diptera). Bull. AMNH *99*, 399–474.

Stern, D.L. (2000). Evolutionary developmental biology and the problem of variation. Evolution *54*, 1079–1091.

Stern, D.L., and Orgogozo, V. (2009). Is genetic evolution predictable? Science *323*, 746–751.

Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirián, L., and Dickson, B.J. (2005). Neural circuitry that governs Drosophila male courtship behavior. Cell *121*, 795–807.

Sucena, E., and Stern, D.L. (2000). Divergence of larval morphology between Drosophila sechellia and its sibling species caused by cis-regulatory evolution of ovo/shaven-baby. Proc. Natl. Acad. Sci. USA *97*, 4530–4534.

Tomoyasu, Y., Arakane, Y., Kramer, K.J., and Denell, R.E. (2009). Repeated co-options of exoskeleton formation during wing-to-elytron evolution in beetles. Curr. Biol. *19*, 2057–2065.

Usui-Aoki, K., Mikawa, Y., and Yamamoto, D. (2005). Species-specific patterns of sexual dimorphism in the expression of fruitless protein, a neural musculinizing factor in Drosophila. J. Neurogenet. *19*, 109–121.

Venken, K.J., He, Y., Hoskins, R.A., and Bellen, H.J. (2006). P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. Science *314*, 1747–1751.

Venken, K.J.T., Carlson, J.W., Schulze, K.L., Pan, H., He, Y., Spokony, R., Wan, K.H., Koriabine, M., de Jong, P.J., White, K.P., et al. (2009). Versatile P[acman] BAC libraries for transgenesis studies in Drosophila melanogaster. Nat. Methods *6*, 431–434.

Villella, A., and Hall, J.C. (1996). Courtship anomalies caused by doublesex mutations in Drosophila melanogaster. Genetics *143*, 331–344.

von Schilcher, F. (1976). The role of auditory stimuli in courtship of Drosophila melanogaster. Anim. Behav. *24*, 18–26.

Wagner, G.P., Pavlicev, M., and Cheverud, J.M. (2007). The road to modularity. Nat. Rev. Genet. *8*, 921–931.

Waldron, I. (1964). Courtship sound production in two sympatric sibling Drosophila species. Science *144*, 191–193.

Yamada, H., Sakai, T., Tomaru, M., Doi, M., Matsuda, M., and Oguma, Y. (2002). Search for species-specific mating signal in courtship songs of sympatric sibling species, Drosophila ananassae and D. pallidosa. Genes Genet. Syst. *77*, 97–106.

Zhang, R., Guo, C., Zhang, W., Wang, P., Li, L., Duan, X., Du, Q., Zhao, L., Shan, H., Hodges, S.A., et al. (2013). Disruption of the petal identity gene APETALA3-3 is highly correlated with loss of petals within the buttercup family (Ranunculaceae). Proc. Natl. Acad. Sci. USA *110*, 5074–5079.